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Original Article

IKBKE influences endometrial cancer growth and increases progestin resistance

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Abstract: The purpose of this study is to investigate the function of IKBKE in cellular autophagy and its potential therapeutic usage in endometrial cancer. In light of the robust correlation observed between IKBKE overexpression and the development, invasiveness, and resistance to drugs in diverse types of cancer, endometrial cancer in particular, this study investigates the mechanism by which IKBKE controls autophagy and its contribution to progestin resistance. The investigation employed a range of cell biology techniques, including MTT, scratch, colony formation, and transwell assays, to examine the impact of CYT387, an IKBKE inhibitor, on cell proliferation and autophagy in the progestin-resistant Ishikawa endometrial cancer cell line in which IKBKE was knocked down. According to the findings, IKBKE silencing significantly inhibited the proliferation, migration, and invasion of endometrial cancer cells, thereby counteracting progesterone resistance. Furthermore, CYT387 effectively inhibited the proliferation and autophagy of these cells. The results of this study underscore the importance of IKBKE in the advancement of endometrial cancer, specifically in its regulation of autophagy and drug resistance. Moreover, they reveal the possibility of employing IKBKE as a target for progestin-resistant endometrial cancer reversal and treatment. This research holds scientific merit as it not only contributes to the comprehension of the molecular mechanisms underlying endometrial cancer but also establishes a foundation for the formulation of new therapeutic strategies - potentially improving treatment efficacy and prognosis for individuals afflicted with endometrial cancer.

Keywords: IKBKE, endometrial cancer, progestin-resistance, autophagy

Introduction

Endometrial cancer, which is common among females, has been extensively treated with progestins like medroxyprogesterone acetate (MPA) as conservative endocrine therapies in young patients diagnosed with early-stage endometrial cancer [1-3]. Progestin resistance has increased in recent years, presenting a difficulty for cancer treatment. According to clinical data, progestin resistance is observed in 30% of young patients diagnosed with endometrial cancer (EC), irrespective of the prescribed medication or treatment protocol. The resistance poses a challenge to treatment and has an adverse impact on the rates of patient survival [4, 5]. Endometrial cancer exhibits a complex array of mechanisms through which progestin resistance develops, including aberrant expression of progestin receptors, dysregulation of signaling pathways, and disruption of the cell cycle [6].

IkappaB kinase-epsilon (IKBKE), a constituent of the IKK family, has been recognized as an oncogenic protein whose expression is elevated in cancers such as breast, ovarian, and prostate [7-11]. Recent research has established a correlation between aberrant IKBKE expression and the development, progression, and resistance to treatment of tumors [12-15]. The investigation of drug resistance mechanisms has yielded mounting evidence that IKBKE is an indispensable component of autophagy and drug resistance processes [16-19].

Autophagy, a highly conserved biological process, transpires when cells engage in lysosomal self-digestion to degrade and recycle internal components. Preserving cellular homeostasis, combating stress, and regulating growth and

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differentiation are all dependent on this mechanism [20]. Autophagy is an essential and balanced process within healthy cells that upholds cellular homeostasis through the engulfment and degradation of organelles, proteins, and other constituents that are aging or malfunctioning. Nevertheless, autophagy has the potential to undergo excessive activation or inhibition under specific pathological circumstances, which may result in cellular imbalance and potentially aid in the development and progression of diseases [21-23]. According to scientific research, IKBKE may regulate cellular autophagy processes via its effect on particular signaling pathways. Abnormal IKBKE expression, being a crucial regulator of autophagy, has the potential to induce pathway activation that disrupts autophagy levels. A further mechanism by which IKBKE may regulate autophagy initiation and vesicle formation is through its influence on the expression of autophagy-related proteins, such as Beclin-1 and LC3 [24-26].

This study endeavors to examine the correlation between autophagy, progestin resistance, and IKBKE in the context of endometrial cancer therapy, with a specific emphasis on the intricate molecular mechanisms.

Methods

Cell culture

Ishikawa cells, which are human endometrial carcinoma cells obtained from Procell Life Science & Technology Co., Ltd. in Wuhan, China, were cultivated using 10% fetal bovine serum in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific). The cells were incubated in a 5% CO_2 environment at 37°C.

Establishment of progestin-resistant EC cell lines

MPA (Sigma-Aldrich Co., St. Louis, MO, USA) was added to progesterone-sensitive endometrial carcinoma cell lines for a duration of 6 months. Monthly increases of twofold in MPA concentration culminated in its attainment of 10 μM [27]. Every 3 days, the medium containing MPA was replaced. After achieving 90% confluence, the viable cells were passaged and subsequently cultured in 10 μM MPA. Cell proliferation was assessed subsequent to drug screening, while signaling pathways were validated in anticipation of the subsequent experiment.

Western blot

For complete lysis, centrifuged and washed cells were lysed on ice for 30 minutes with a suitable volume of lysate (including PMSF). Following this, 30 μg of the protein extract was deposited onto a polyacrylamide gel with a 15% concentration. Following the separation of proteins via electrophoresis, the separated proteins were transferred from the gel to a PVDF membrane. At room temperature for 15 minutes, the membrane was blocked with a rapid blocking solution (Wuhan Sevier Biotechnology Co., Ltd., Wuhan, China). Following this, 16 hours at 4°C were spent applying antibodies against PR (5264; Zen Bio, Chengdu, China), IKBKE (3416; Cell Signaling Technology), NF-κB p65 (8242; Cell Signaling Technology), phosphorylated NF-κB p65 (Ser536; 3033; Cell Signaling Technology), LC3B (3868; Cell Signaling Technology), ATG5 (2630; Cell Signaling Technology), Beclin-1 (3495; Cell Signaling Technology), MDR1 (13342; Cell Signaling Technology), and β-actin (ac-026; ABclonal, China). Following that, rabbit anti-mouse IgG conjugated with specific horseradish peroxidase was introduced onto the membrane, followed by a one-hour incubation period at room temperature. Utilizing an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA), chemiluminescence was identified.

Cell proliferation assay

A low density (5×10^3 cells) of cells was inoculated into 96-well plates. At 0, 24, 48, and 72 hours, ten microliters of 3-4.5.2-2.5-(MTT; 5 mg/ml; BioFroxx) were added to each well. After introducing 100 μl of dimethyl sulfoxide (DMSO; BioFroxx), the absorbance at 492 nm was measured using a 96-well plate reader.

Cell migration assay

The capacity for cell migration is assessed using a scratch assay. At the outset, cells were cultured until they attained an appropriate density in a 6-well plate. Following the formation of a homogeneous cell monolayer, a linear scratch was performed utilizing a 200-μl pipette tip. Following a gentle PBS wash to eliminate unattached cells, the cells were supplemented with

serum-free culture medium. In order to monitor cell migration, images of the scratch area were captured at predetermined time points. The assessment of cell migration ability was conducted by measuring the degree of scratch closure. To assist in simulating the physiological milieu, this experiment was carried out in serum-free conditions.

Cell invasion assay

Cell migration capacity is investigated using the Transwell assay. To simulate in vivo conditions, cell suspensions were added to the upper layer of the Transwell with pre-filled medium at the bottom and chemical treatments or drugs added as needed. Cells were incubated at 37°C and 5% CO_2 for a period of time. At the end of the assay, non-migrating cells in the upper chamber were removed, and the remaining cells were fixed and stained with crystal violet. Migrating cells were observed under a microscope and quantified. This assay facilitates the study of cell migration by providing an objective assessment of the ability of cells to migrate.

Colony formation assay

The colony-formation assay is a critical technique utilized to assess clonogenic capacity and cellular proliferation. At the outset, a suitable quantity of cells (specifically, 200 cells) are suspended within the culture medium. For 2-3 weeks, at 37°C and 5% CO_2, the cells are cultured until visible colonies form. Following cultivation, the medium is removed, and the cells are air-dried after being washed with PBS. Following this, the colonies are observed through the application of formaldehyde fixation and crystal violet staining. By quantifying the quantity and dimensions of colonies, one can evaluate cellular proliferation and clonogenic potential quantitatively.

Transfection with mRFP-GFP-LC3 adenovirus and fluorescence microscopy detection were employed to assess autophagy

In a 24-well plate containing coverslips, cells were seeded at a density of 1×10^4 cells per well. After cells had adhered, the mRFP-GFP-LC3 adenovirus was introduced and left to incubate for a duration of 12 hours. Following medium replacement, the cells were subjected to a 24-hour treatment of MPA (10 μM). Following fixation with 4% paraformaldehyde and PBS washing, cells were stained for 3-5 minutes at room temperature with DAPI (BL105A) nuclear dye, rinsed with PBS, and subsequently mounted using 30% glycerol. Fluorescence microscopy was utilized to examine autophagy; puncta in yellow denoted autophagosomes, whereas puncta in red indicated autolysosomes.

Lentiviral-infected cells

Having been obtained from Shanghai Heyuan Biology Co., Ltd., the IKBKE knockdown lentivirus was synthesized for packaging purposes. It displayed resistance to purines and had the following sequence: 5'-GCATCATCGAACGGCTAAATA-3'; The NC group sequence of IKBKE shRNA is: 5'-CCTAAGGTTAAGTCGCCCTCG-3'. The day prior to transfection, cells were inoculated into 24-well plates and allowed to grow overnight, until they had reached 20-30% confluence. After a period of 48 hours, puromycin was introduced into the cells at a final concentration of 8 μg/ml. After a week of maintaining this concentration, further experiments were carried out following transfection.

Statistical analysis

The analysis of the data was performed utilizing SPSS 16.0. Results from three separate independent experiments are shown as mean ± SD for the cell experiment. One-way analysis of variance (ANOVA) was implemented. In order to compare the means of two groups, a t-test was utilized. When factorial designs were employed to compare data across groups, analysis of variance was utilized. A *P*-value below 0.05 was deemed to indicate statistical significance.

Results

Progestin-resistant cells exhibit increased cell growth and autophagy

The development of progestin-resistant cell lines was achieved through prolonged treatment with medroxyprogesterone acetate (MPA). Progestin-resistant Ishikawa cells exhibited lower expression of the progesterone receptor (PR) (**Figure 1A**). Progesterone-resistant Ishikawa cells display a significant level of MDR1 (Multidrug Resistance Protein 1) expression, a drug resistance marker (**Figure 1B**). The prolif-

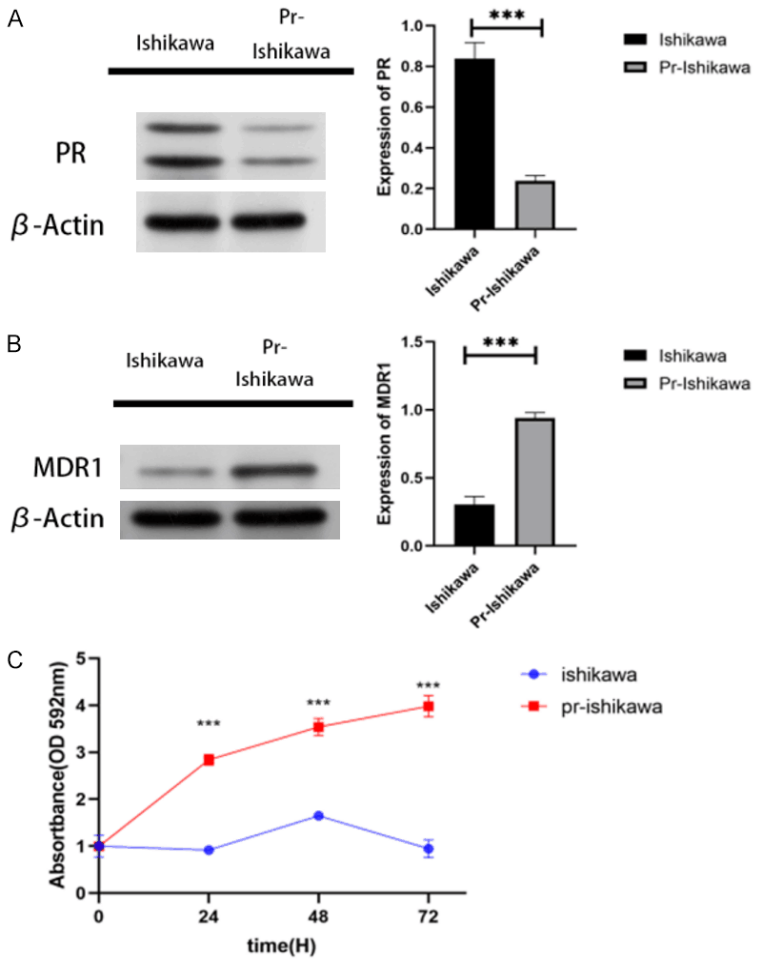

Figure 1. Progestin-resistant cells exhibit enhanced cell proliferation. Note: A. Western blot depicting the expression of PR. B. Western blot illustrating the expression of MDR1. C. MTT assay revealing cell growth curves of Ishikawa progenitor and progestin-resistant cells. *P<0.05, **P<0.01, ***P<0.001.

eration of progestin-sensitive and progestin-resistant Ishikawa cells in response to MPA was assessed using the MTT assay. The MTT results demonstrated that MPA inhibited proliferation significantly in progestin-sensitive Ishikawa cells but not in progestin-resistant cells (**Figure 1C**). These results suggest that prolonged progestin therapy induces PR downregulation, which in turn promotes endometrial cancer cell growth.

IKBKE inhibits the growth of progesterone-resistant endometrial cancer cells

Using WB and RT-PCR assays, the expression levels of IKBKE in progesterone-sensitive and progesterone-resistant Ishikawa cells were determined (**Figure 2A**, **2B**). Stable cell lines were generated through the transfection of progesterone-resistant cell lines with upregulated IKBKE expression with the IKBKE shRNA lentivirus. In contrast to the unintentional sequence group, the IKBKE shRNA lentivirus group demonstrated a substantial decrease in the levels of both IKBKE protein and mRNA (**Figure 2C**, **2D**).

MTT and colony formation assays demonstrated that IKBKE knockdown in progesterone-resistant cells significantly decreased cell proliferation in the experimental group relative to the control group (P<0.001) (**Figure 3A**, **3B**). The scratch assay demonstrated that the knockdown of IKBKE substantially retarded the migration rate of drug-resistant cells, resulting in a substantial reduction in the rate of wound healing when compared to the control group (P<0.001) (**Figure 3C**). The findings were additionally corroborated by the Transwell assay results, which illustrated that the knockdown of IKBKE substantially diminished the migratory and invading capacities of cells (P<0.001) (**Figure 3D**). The cumulative findings unequivocally illustrate the significant inhibitory effect that IKBKE silencing exerts on drug-resistant cell proliferation, migration, and invasion.

IKBKE influences autophagy in progesterone-resistant endometrial cancer cells

MPA promoted autophagy in progesterone-resistant cells, as demonstrated by autophagy-tagged protein blotting (**Figure 4A**). Furthermore, autophagy levels decreased substantially following transfection with the sh-IKBKE lentivirus (**Figure 4B**). Using fluorescence microscopy, mRFP-GFP-LC3 adenoviral transfection, which is employed to identify cellular autophagy, unveiled multiple bright yellow fluorescent

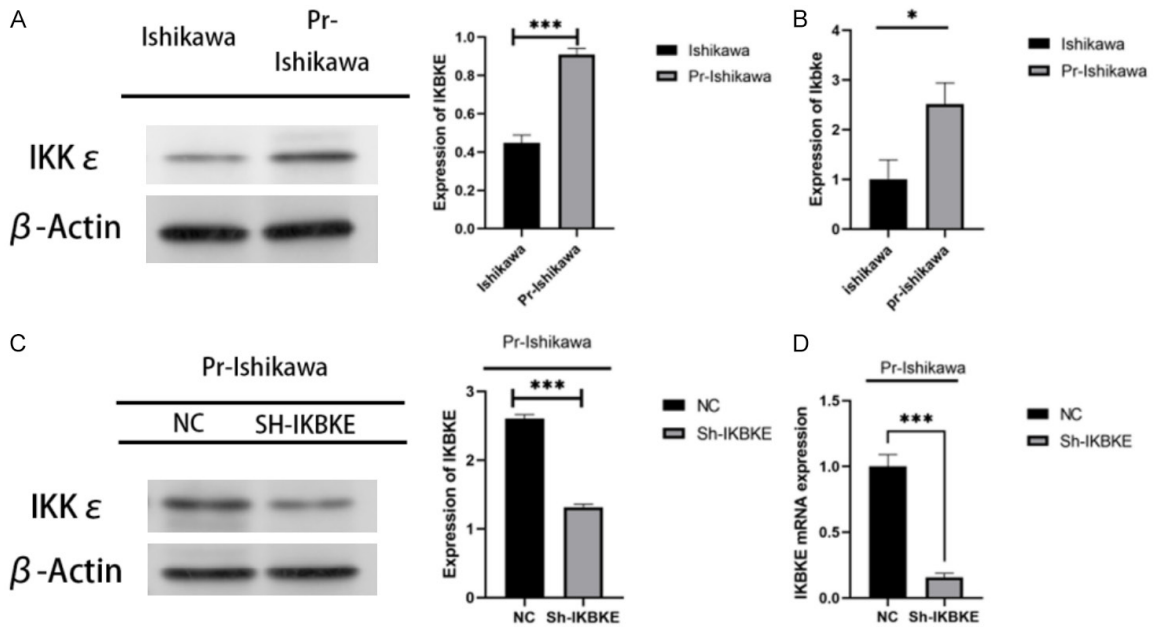

Figure 2. IKBKE influences the growth of progesterone-resistant endometrial cancer cells. Note: A, B. The expression of IKBKE protein and mRNA in the two cell lines. C, D. PCR and WB analysis of IKBKE mRNA and protein levels in progesterone-resistant Ishikawa cell lines post-transfection with sh-IKBKE lentivirus.

spots within progesterone-resistant cells. Following IKBKE gene silencing, there was a significant decrease in fluorescence and autophagy (**Figure 4C**).

Impact of CYT387 on proliferation and autophagy in progesterone-resistant endometrial cancer cells

The effect of CYT387, an autophagy inhibitor that targets IKBKE, on the growth of EC cells and autophagy in progesterone-resistant Ishikawa cells was investigated. To ascertain the proliferation of progesterone-resistant cell lines that had been treated with CYT387, MTT was performed. The cell lines exhibited a substantial reduction in proliferation rate subsequent to CYT387 treatment, suggesting a substantial diminishment in their proliferative capability (**Figure 5A**). The utilization of autophagy labeling in protein blotting demonstrated a substantial decrease in autophagy level subsequent to cyt387 treatment (**Figure 5B**). These findings suggest that treatment with CYT387 inhibits the proliferation of progesterone-resistant endometrial cancer cells and reduces autophagy.

Discussion

Progesterone is an essential therapeutic agent for endometrial cancer due to its ability to adhere to progesterone receptors and impede cell proliferation. However, progesterone-resistant endometrial cancer cells proliferate in response to MPA treatment [28]. As a consequence, we formulate the hypothesis that progesterone might augment the proliferation of progesterone-resistant endometrial cancer cells via mechanisms other than the PR pathway.

IKBKE, an atypical constituent of the IKK family, is implicated in inflammatory responses and metabolic diseases [29]. Recent research from both domestic and international sources has documented a substantial overexpression of IKBKE in malignant gliomas, which exhibits a positive correlation with the malignant nature of gliomas. IKBKE facilitates the proliferation, invasion, and migration of glioma cells, thereby contributing to their in vivo development. Additionally, IKBKE serves as a therapeutic target for late-stage prostate cancer (PC). By specifically targeting IKBKE, one can circumvent resistance to androgen receptor (AR)-targeted therapy, thereby impeding the ability of cells to proliferate, migrate, and form colonies. Furthermore, a multitude of malignancies, including gastric cancer, breast cancer, esophageal cancer, endometrial cancer, prostate cancer, lung squamous cell carcinoma, and pancreatic cancer, exhibit a higher level of

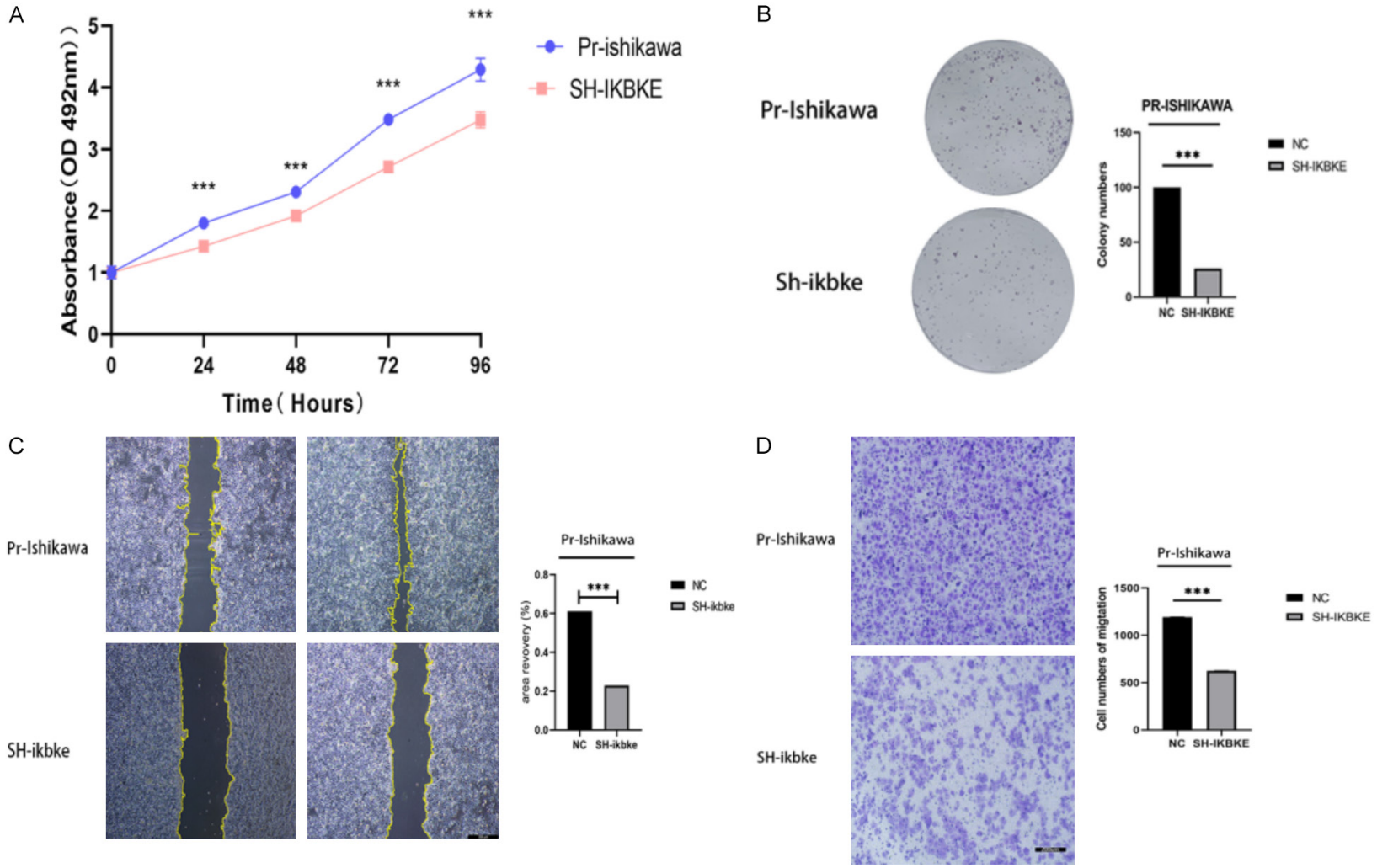

Figure 3. Effect of silencing IKBKE gene on the growth of progesterone-resistant endometrial cancer cells. Note: A. MTT assay illustrating the growth curve of progesterone-resistant cell lines post-transfection with sh-IKBKE lentivirus. B. Colony formation assay indicating significant inhibition of proliferation in progesterone-resistant cells post-transfection with sh-IKBKE lentivirus. C. Scratch assay demonstrating a significant decrease in cellular healing in cells post-transfection with sh-IKBKE lentivirus. D. Transwell assay revealing that IKBKE knockdown substantially reduces cell migration and invasion. *P<0.05, **P<0.01, ***P<0.001. Bar = 200 μm.

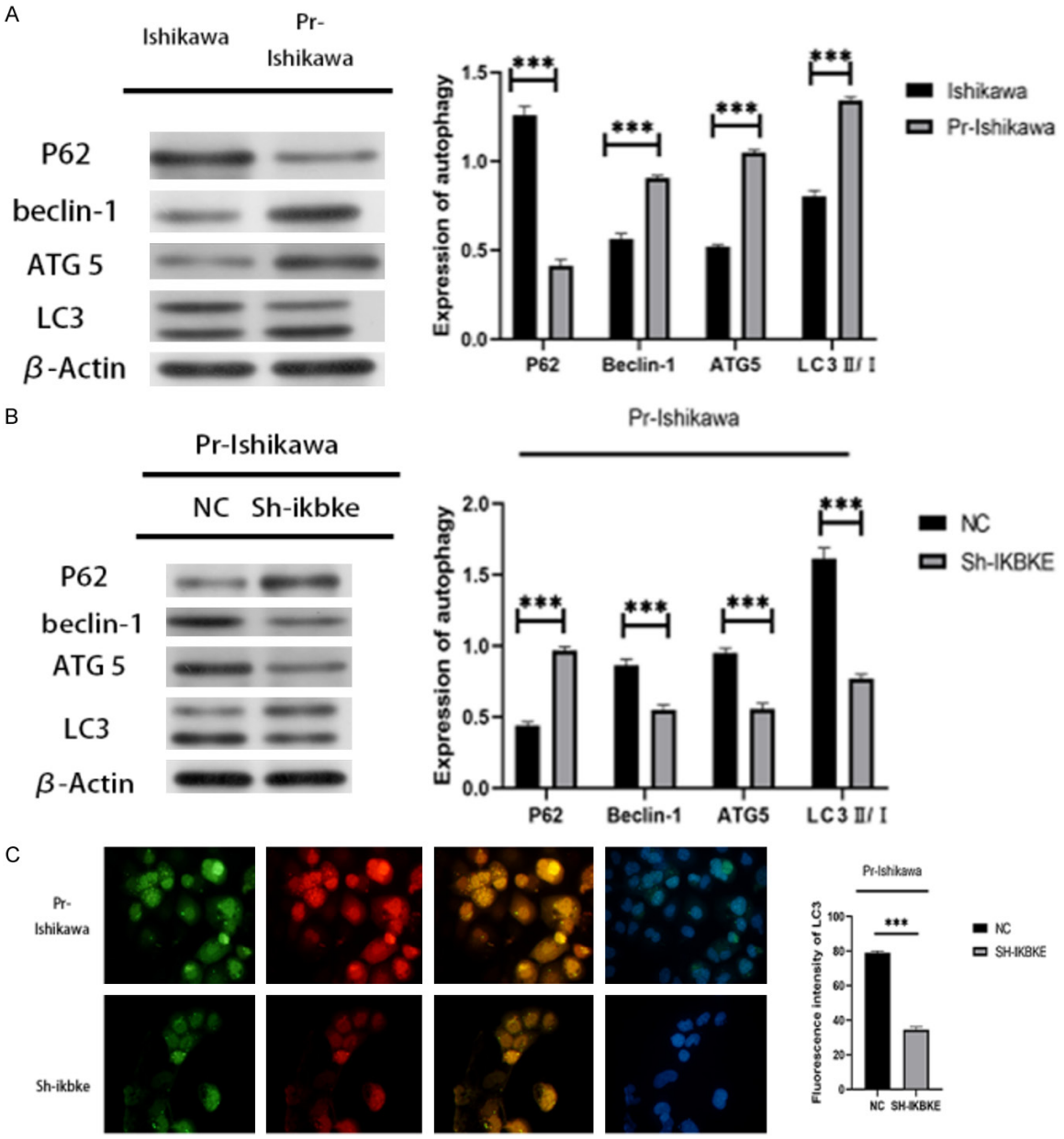

Figure 4. IKBKE influences autophagy in progesterone-resistant endometrial cancer cells. Note: A, B. Protein blotting illustrates the expression of autophagy markers Beclin-1, P62, LC3B, ATG5, and β-actin as an internal control. C. Fluorescence microscopy used to detect changes in autophagy post mRFP-GFP-LC3 adenovirus transfection in cells. *P<0.05, **P<0.01, ***P<0.001. Bar = 50 μm.

IKBKE expression. Moreover, breast cancer and non-small-cell lung cancer are capable of developing endocrine resistance when IKBKE expression is elevated [30-32]. MPA was applied to progesterone-sensitive and progesterone-resistant Ishikawa cells, which had been transfected with LV-IKBKE and sh-IKBKE lentivirus, respectively, in this portion of the experimental study. Experimental procedures utilizing MTT, scratch, colony formation, and Transwell assays revealed that, in comparison to the control group, the shIKBKE group exhibited a substantial inhibition of proliferation, migration, and invasion. This finding suggests that the reversal of progesterone resistance can be achieved through the silencing of IKBKE.

MPA treatment was applied to progestin-sensitive and progestin-resistant Ishikawa cells that had been transfected with LV-IKBKE and sh-

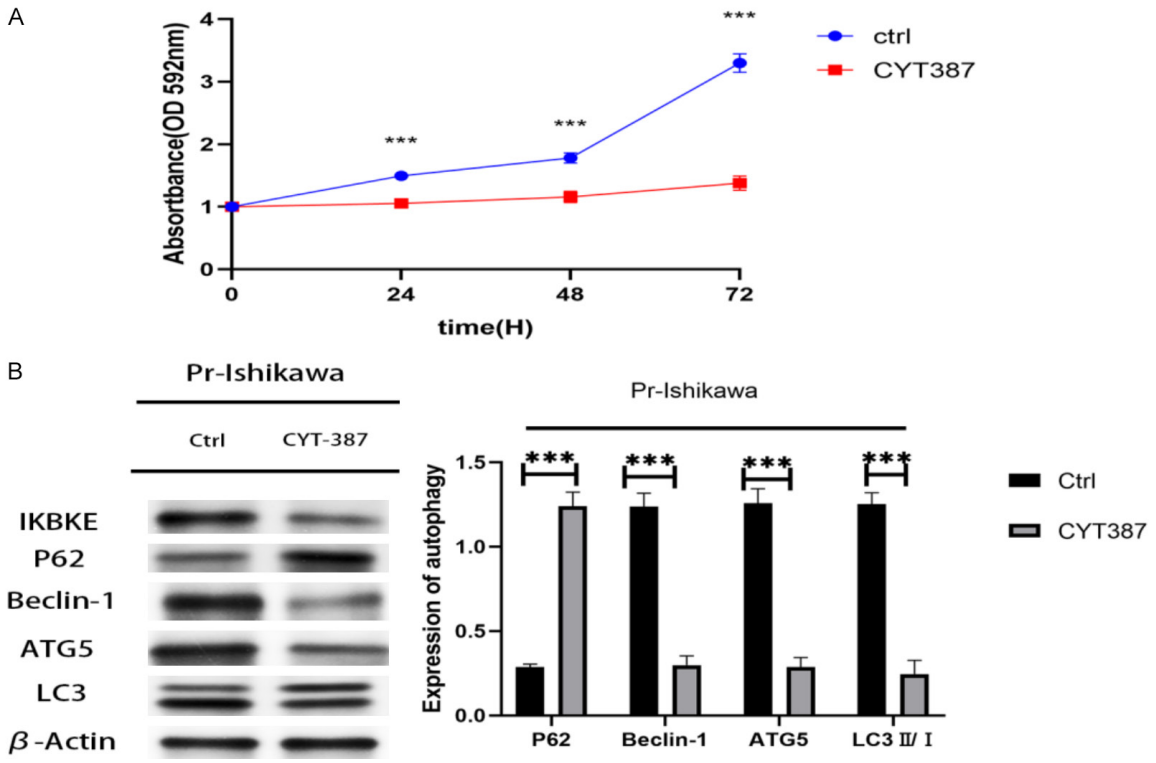

Figure 5. CYT387 suppresses the proliferation of progesterone-resistant endometrial cancer cells. Note: A. MTT assay displaying growth profiles of resistant cell lines post-CYT387 treatment. B. Protein blotting illustrates the expression of autophagy markers Beclin-1, P62, LC3B, ATG5, and β-actin as an internal control. *P<0.05, **P<0.01, ***P<0.001.

IKBKE lentiviruses, respectively, in this experimental study. The reversal of progestin resistance by silencing IKBKE was demonstrated by markedly inhibited proliferation, migration, and invasion capabilities in the shIKBKE group relative to the experimental group, as determined by MTT, scratch, colony formation, and Transwell assays.

An overexpression of IKBKE has the potential to induce autophagy and facilitate the proliferation of cancer cells. Autophagy is essential for tumor growth in numerous types of cancer, according to accumulating evidence. It satisfies the tumors' high metabolic demands and promotes resistance to a variety of treatment-induced stresses [33, 34]. This paper centers on a novel mechanism through which CYT387, a potent IKBKE inhibitor, impedes the progression of endometrial cancer. Cellular autophagy is induced by IKBKE overexpression in breast cancer, particularly triple-negative breast cancer (TNBC), according to Barbie. An IKBKE inhibitor, CYT387 (also known as mometinib), suppressed the proliferation of triple-negative breast cancer by impeding the activation of NF-κB and STAT induced by IKBKE [35]. Additionally, CYT387 was administered to renal cell carcinoma, myelone proliferative necrotic, and bone marrow proliferative tumors [36, 37]. In renal cell carcinoma, the combination of CYT387 and dasatinib decreases cell proliferation while increasing apoptosis. The proliferation of non-small-cell lung cancer resistant to epidermal growth factor receptor inhibitors was inhibited significantly by the combination of cetuximab and CYT387 [38]. The findings of this research indicate that the administration of CYT387 suppresses autophagy and impedes the growth of progesterone-resistant endometrial cancer cells. As a result, we postulated that IKBKE stimulated additional translational pathways in order to promote endometrial cancer autophagy and progression.

This study has several limitations. For instance, while there are numerous prevalent human endometrial cancer cell lines, it would be more convincing to replicate the investigation using different cell lines. Furthermore, to enhance

the rigor of the mechanistic research, it would be beneficial to incorporate the downstream pathway.

Conclusion

In summary, this study investigates the mechanisms by which IKBKE regulates the proliferation of endometrial cancer and augments resistance to progesterone. The results of this study provide novel insights into the pathophysiological mechanisms underlying endometrial cancer and lay the groundwork for future developments in more targeted therapeutic approaches.

Disclosure of conflict of interest

None.

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